Single Molecule Pointillism

Challenges in Localization Based Super-Resolution Microscopy

Single molecule super-resolution imaging enables microscopists to go beyond the resolution limit set by optical diffraction, and to image structures much smaller than the wavelength of the probing light. Using variants of traditional microscopes, it reveals the previously unseeable, with the potential to revolutionize our understanding of the nanoscale structure of biological samples. However, this method is still under intensive development and capturing super-resolved, multicolour, 3D images in fixed or live cells remains a scientific and technical challenge.

Optical microscopy techniques, and particularly fluorescence approaches, such as TIRF, confocal and confocal two-photon microscopy, are essential techniques in modern biology. They permit the study of molecular complexes and subcellular organelles in the context of cell cultures and model organisms with minimal invasiveness, very high sensitivity and excellent selectivity.

The use of molecular labelling methods such as dye immunolabelling and genetically encoded fluorescent proteins [1] permits functional properties to be measured with a wide range of information available about the temporal and spatial scales of the system studied at subcellular resolution. However, the spatial resolution of traditional optical imaging techniques is limited by optical diffraction, typically to around half of the wavelength of the emitted light (250 nm), which is larger than the scale of much of the subcellular machinery.

Exploring this nanoscale world requires much more sophisticated equipment with a spatial resolution approaching the molecular scale. Several methods [2] referred to as "super-resolution" microscopies, have been developed over the last few years, which have overcome this resolution limit.

Localization Based Super-Resolution Microscopy

Single molecule localization microscopy is a new class of super-resolution techniques able to reduce resolution down to the 10 nm scale. This approach is sometimes referred to as pointillism, by analogy with the style of painting where images are built up from colored dots - for reasons that should become obvious.
The techniques make use of either photoactivatable fluorescent proteins or commercial organic dyes, and can be operated in 3D and multicolor modes too.

The different approaches of localization based super-resolution techniques (PALM [3], FPALM [4], STORM [5], and dSTORM [6]) apply precise point spread function (PSF) fitting to the images of spatially and temporally separated fluorescent molecules. In other words, it is possible to measure the location of each fluorescent molecule with sub-diffraction precision so long as each fluorophore does not overlap another diffraction-limited fluorophore.

Therefore by using photoactivatable fluorescent proteins, which are rapidly activated and bleached, or dyes which can be induced to 'blink' so that only a minority of the molecules are active in any single camera frame, it is possible to bypass the diffraction limit. The price of this greatly increased resolution is that enough camera frames have to be acquired to image a significant proportion of the fluorescent molecules, which typically means in the order of thousands or tens of thousands of frames. Since the localization precision strongly depends on the detected number of photons emitted by a single molecule, these wide-field methods typically use high sensitivity, low noise cameras together with bright fluorescent molecules. Although the principles of photophysics behind the blinking mechanism can significantly differ, the instrumentation and software requirements are very similar.

**Instrumentation**

Localization-based microscopy systems are typically modified wide field inverted fluorescence platforms able to detect single fluorescent molecules. Total internal reflection (TIRF) illumination [7] can drastically reduce the fluorescence background by exciting molecules only in the boundary layer in the vicinity (150 nm) of the coverslip-sample interface. This makes it ideal for imaging processes in the cell surface membrane either in fixed or live cells. Highly inclined illumination
can reveal the deeper intracellular sections up to 15-20 microns, but at a reduced signal to noise ratio, and consequently with less precise localization.

In practice, one of the most critical issues is the motion of the sample relative to the microscope objective. This thermal or mechanical drift can arise from different sources such as the cooled detector, the airflow and vibration around the frame, and also the stage itself, which can drift during the measurement time (several minutes). The amplitude of this time-dependent drift is typically below the optical resolution and only causes problems in super-resolved imaging. Autofocus systems can keep the samples in focus with high precision, and fiducial markers can be also applied to register the captured frames. Ideally, a single marker in the field of view will suffice, because the drift does not depend on the position. However, static but wavelength- and position-dependent optical offsets can affect the final image, and lead to misinterpretations such as co-localization errors in multicolor imaging.

Localization microscopy techniques typically fit a 2D Gaussian distribution to the measured point spread function. The principal reason for fitting a Gaussian distribution is its simplicity (x and y coordinates can be separated) and speed (existing quick algorithms can be applied). Although the theoretical PSF is not truly Gaussian, it is a very good approximation, especially at a high NA [9]. The Gaussian fitting generally works well, since the only physical parameter we want to determine is the central position of the measured PSF, which is assumed to be the exact position of the fluorescent molecule. The real shape of the PSF is typically not taken into consideration, but it cannot be easily determined either, since the pixel size of the applied CCD camera is matched to the standard deviation of the PSF [10]. In other words, the PSF is measured with a very poor spatial resolution precluding the determination of its fine structure. However, the center of a distorted PSF is shifted depending on the wavelength, and hence it can cause a slight error during co-localization on a two-color STORM image.

In short, a cooled camera directly attached to the microscope frame makes the system thermally unstable, and the drift of a single bead (or quantum dot) can be comparable with the localization precision only after several hours (fig. 1a). Defocus can also shift the localization because of a slight asymmetry of the defocused PSF (fig. 1b). The illumination mode does not affect the localization, since in fluorescence microscopy the PSF basically depends on the detection path of the system. Chromatic aberration can also introduce a slight offset between the different channels (fig. 1c). We have to note that the amplitude of the above mentioned potential drifts and offsets are typically below 100 nm, and they may cause problems in conventional optical microscopes only when they are applied as super-resolved microscopes. For the minimization and elimination of these error
sources drift correction hardware, post processing algorithms and calibration procedures have been proposed and widely used. Time dependent mechanical drift can be minimized and corrected using autofocus systems, and simultaneously by imaging fiducial markers (beads, quantum dots, gold particles, etc.). Static optical offsets can be eliminated by the precise calibration of the imaging system.

**One Colour Single Molecule Imaging *in Vitro* and *in Cells***

Microtubules and actin filaments formed on a coverslip *in vitro* and labelled by organic dye molecules (Alexa, HiLyte, ATTO, etc.) are the most widely used test samples, since their preparation is relatively simple, and their sub-resolution diameter is well known. In case of actin filaments (fig. 2a) the measured width is affected by the size of the fluorescently labelled Phalloidin bound to the actin. The measured image can be deconvolved by the known real width to determine the effective PSF (related to the localization precision) of the STORM system. Furthermore, this effective PSF can be used to determine even more accurately the size of unknown objects such as *in vitro* amyloid fibrils (fig. 2b) [11] and vesicles formed in the cell membrane (fig. 2c).

One can even image deeper sections in a cell by using highly inclined illumination (fig. 2d), but the increased fluorescent background reduces the localization precision and hence the final image quality. In single color imaging the ideal switching buffer condition can be optimized and matched to the applied dye molecule. However, multi-color imaging requires different dyes "working" well in the same buffer conditions and chromatic offset also becomes a central issue.

**Multi-color Imaging Strategies**

The ability to label different molecules with different dyes and image them is an extremely powerful approach used by cell and molecular biologists and biomedical researchers. Multicolor imaging can reveal molecular interactions (dimer formation, protein aggregation etc.) and intracellular traffic pathways. The two (or more) images can be captured sequentially [12, 13] or simultaneously [14, 15]. In multicolor imaging the applied dyes have to properly work under the very same photoswitching environment (buffer), illumination mode and sometimes intensity.

Several dye (STORM/STORM) and organic dye/fluorescent protein (STORM/PALM) combinations have been proposed in the last few years and the potential advantages were clearly demonstrated. However, the interpretation of multicolor localization based super-resolved images requires extra care. Optical offset as mentioned above can introduce a slight drift between the two images - as can be seen in figure 3 - and lead to misinterpretation of the reconstructed image.
Calibration of the optical setup using standard samples (actin filaments etc.) is necessary.

**Conclusion**

The greatly improved resolution of super-resolution microscopes creates some new challenges and opportunities for instrumentation developers and users alike. We need well-calibrated stable microscopes with minimal drift and chromatic offsets, cameras that combine high sensitivity with high frame rates, dyes and fluorescent proteins which are brighter and with controllable fast photoswitching and user-friendly software for acquiring and analyzing datasets. Success in this should make it a routine matter to take super-resolution images with resolutions approaching 10 nm.

**Acknowledgement**

This work was funded by a Welcome Trust/MRC Strategic Award for Neurodegenerative Disorders, and supported by grants from the EPSRC and the BBSRC.

**References**


For further literature please ask the authors.

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